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Full Length Research Paper

Influence of cultivation conditions on the production of a protease from *Aspergillus carbonarius* using submerged fermentation

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The ability of a raw starch digesting amylase producer, *Aspergillus carbonarius* for protease production was evaluated in this study using standard methods. The fungus grew and produced appreciable levels of protease using different carbon and nitrogen sources. Sources of carbon and nitrogen significantly ($p < 0.001$) influenced protease production by this fungus. Soybean meal and glucose were the best nitrogen and carbon sources, respectively for the production of protease by *A. carbonarius*. Maximum protease production was achieved with 5% glucose and 5% soybean meal in combination with 0.1% peptone (as carbon and nitrogen sources, respectively), 0.04% $FeSO_4$, 0.1% NaCl, 0.1% (v/v) Tween 80 and initial pH 6.0. Time course of enzyme synthesis by the fungus showed that the enzyme production occurred through the logarithmic to stationary growth phases with maximum enzyme yield being obtained on the 9th day of fermentation corresponding to the final culture pH of 4.6. The results suggested the potential secretion of protease by *A. carbonarius* using cheaper and locally available substrates as carbon and nitrogen sources and its subsequent application in various industries.

Key words: *Aspergillus carbonarius*, glucose, soybean and peptone, protease, production.

INTRODUCTION

Proteases (EC3.4.21-24) are a single class of enzymes occupying a pivotal position with regard to their commercial and physiological usage (Rao et al., 1998; Chellapandi, 2010). Proteases constitute one of the three largest groups of industrial enzymes (of which 95% are hydrolytic enzymes) and amounts to about 30 to 65% of the total worldwide enzyme production and sales (Rao et al., 1998; Germano et al., 2003; Tunga et al., 2003; Ogbonna et al., 2004; Thys et al., 2006; Chellapandi, 2010). Proteases have a large variety of applications in different industrial processes in the food, medicine, pharmaceutical, detergent, leather and silk industry and in the recovery of silver from x-ray films (Anwar and Saleemuddim, 2000; Sookkheo et al., 2000; Feng et al., 2001; Adinarayana and Ellaiah, 2002; Son and Kim, 2002; Tunga et al., 2003; Pastor et al., 2004). They are also used as enzyme debriders and as a catalyst for the recovery of N-protected amino acids with unusual side chains (Anwar and Saleemuddim, 2000).

Proteases are grossly subdivided into two major groups: exopeptidases and endopeptidases depending on their site of action. Exopeptidases cleave the peptide bonds proximal to the amino or carboxyl termini of the substrate, whereas endopeptidases cleave peptide bonds distant from termini of the substrate. Based on the functional groups present at the active site, proteases are Further classified into four prominent groups: serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Son and Kim, 2002, Beynon and Bond, (1989). Microorganisms are the most essential source for enzyme production because of their rapid growth, the limited space required for their cultivation, and the ease

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with which they can be generally manipulated to generate new enzymes with altered properties (Rao et al., 1998; Kumar et al., 2008). Proteases are secreted by a wide range of microorganisms which include moulds, yeasts, bacteria, fungi and mammalian tissues (Tunga et al., 2003). At present majority of commercially available proteases are secreted by *Bacillus* spp., although there have been increasing reports of the potential use of proteases of fungal origin (Andrade et al., 2002; Papagianni et al., 2002; Germano et al., 2003; Tunga et al., 2003; Jitender et al., 2006; Hajji et al., 2007; Paranthaman et al., 2009; Chellapandi, 2010; Soares et al., 2010; Vishwanatha and Appu, 2010).

Filamentous fungi are capable of utilizing a wide range of carbon and nitrogen sources for the production of different hydrolytic enzymes in copious amount into their environment. However, fungi as enzyme producers have many advantages considering that the produced enzymes are normally extracellular, making easier its recovery from the fermentation broth (Germano et al., 2003; Jitender et al., 2006). The use of fungal enzymes is safer than the use of bacteria since they are normally recognized as generally regarded as safe (GRAS) (Germano et al., 2003). In commercial practice, the optimization of medium composition is done to maintain a balance between the various medium components thereby reducing the amount of unutilized components at the end of fermentation. Recently, researchers have directed their effort towards evaluating the effect of different carbon and nitrogen nutrient cost-effective substrates on the yield of enzymes, requirement of divalent metal ions in the fermentation medium and optimization of environmental and fermentation parameters (Adinarayana and Ellaiah, 2002). Furthermore, there has been no established defined medium for the optimum production of protease from various microbial sources, each organism possesses its own peculiar condition for optimum protease production.

As the need for proteases increases for industrial processes, there is need to search for fungal protease producers in the presence of economical indigenous carbon and nitrogen sources. The yield of extracellular enzymes is significantly influenced by physicochemical conditions (Srinubabu et al., 2007a; Kalaiarasi and Sunitha, 2009). Hence physical parameters are optimized for the maximum production of protease. In this study, therefore, we report the influence of cultivation conditions on the production of a protease from *Aspergillus carbonarius* using submerged fermentation.

EXPERIMENTAL

Microorganism

A. carbonarius (Bainier) IMI 366159, originally isolated from rotten cassava tubers (Okolo et al., 1995), was used for this study. The organism was maintained on potato dextrose agar (PDA) slants at 4°C and subcultured every 6 months.

Screening for proteolytic activity

The ability of *A. carbonarius* to secrete protease was carried out according to modified method (Saran et al., 2007). The protease detection medium was prepared with 10 g of skim milk and 10 g of agar-agar each dissolved in 100 ml distilled water, and 300 ml of 0.1 M citrate phosphate at pH 6.0. They were separately autoclaved at 15 psi for 20 min to avoid coagulation and charring of milk and mixed aseptically and poured when it was hot. The plates were inoculated with *A. carbonarius* and incubated for 96 h at 30°C. Ten (10) percent tannic acid was flooded on the milk agar plate for appearance of zone of hydrolysis. A clear zone around the colony indicated protease activity.

Preliminary production of enzyme

A preliminary study for the production of protease in submerged fermentation was carried out using a basal medium. The enzyme preparation was obtained by inoculating two loopfuls of agar plugs (1.7 cm in diameter) of 96 h old profuse growth into 250 ml Erhlenmeyer flasks each containing 100 ml of sterile fermentation medium. The basal fermentation medium with a composition of 1.5% peptone, 1% $(NH_4)_2HPO_4$, 0.1% $MgSO_4$, and 2% raw cassava starch with pH 3.0 (Medium A) was used as the original medium for *A. carbonarius* to check for protease production. Fermentation was carried out in an orbital-shaking incubator (Gallen Kamp Orbital Shaker) at 100 rpm for 6 days at 30°C. After fermentation, mycelial pellets were separated by filtration through Whatman No.1 filter paper at 4°C. The cell-free filtrate was used to assay for protease activity.

Enzyme assay

Extracellular proteolytic activity was determined according to the modified method of (Anson, 1938) as described in (Berla and Suseela, 2002) using casein as the substrate. The reaction mixture contained 1 ml of 1.5 (w/v) casein in 0.1 M citrate phosphate buffer (pH 6.0) and 1 ml of culture supernatant. The mixture was incubated at 40°C for 30 min. The enzyme reaction was terminated by addition of 6 ml of 5% (w/v) trichloroacetic acid (TCA). The mixture was allowed to stand for 10 min and filtered through Whatman No. 1 filter paper. To 1 ml of filtrate, 3 ml of 0.5 M Na_2CO_3 solution and 1 ml of 3-fold diluted Folin-Ciocalteau reagent (BDH) Chemicals Ltd, Poole England) were added and mixed thoroughly. A blank was prepared as described previously except that the TCA solution was added before the enzyme. The colour developed after 30 min of incubation at 40°C was measured in a Unicon UV-2102 PC spectrometer at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine from casein per minute at 40°C under the assay conditions described previously. All assays were carried out in duplicate and mean values reported.

Effect of carbon substrates

The effects of carbon sources 1.5% (w/v) on protease production were investigated by replacing cassava starch used in the basal medium for preliminary study with starches from plantain, yam, maize (corn), sorghum, potato, cocoyam, rice, soluble starch and glucose in the basal medium. Fermentation was carried out in an orbital-shaking incubator at 100 rpm for 6 days at 30°C. Following the termination of fermentation, the culture broth was filtered through Whatman No.1 filter paper at 4°C and filtrate recovered. The cell-free filtrate was used to assay for protease. Apart from glucose and

soluble starch other carbon substrates were prepared in our laboratory as described in our previous report (Okolo et al., 2000). Soluble starch and glucose were obtained from Merck Chemicals (Darmstadt, Germany).

Effect of nitrogen substrates

The following nitrogen substrates 1.5% (w/v) were used to evaluate the influence of organic and inorganic nitrogen sources on protease production by the fungus: soybean meal, bambara meal, bean meal, melon meal, fish meal, cowpea meal, casein, ammonium orthophosphate, potassium nitrate and ammonium sulfate. The respective nitrogen sources were added as a sole source of nitrogen (1.5%, w/v), in place of peptone and $(NH_4)_2HPO_4$ employed in the basal medium. Fermentation was carried out in an orbital-shaking incubator at 100 rpm for 6 days at 30°C. Following the termination of fermentation, the culture broth was filtered through Whatman No.1 filter paper at 4°C and filtrate recovered. The cell-free filtrate was used to assay for protease. All the organic nitrogen substrates were prepared in our laboratory according to standard procedures. All the inorganic nitrogen compounds used in this work were of analytical grade.

Effect of concentration of nitrogen source

The effect of varying concentration (0.5 to 6% w/v) of the best nitrogen source (soybean meal) in the fermentation medium for the production of protease was evaluated. Two agar plugs (1.7 cm in diameter) of profuse growth of the *Aspergillus* culture cultivated on PDA for 96 h at room temperature were inoculated into 250 ml Erlenmeyer flasks each containing 100 ml sterile fermentation medium with varying concentration (0.5 to 6% w/v) of soybean. Fermentation was carried out in an orbital-shaking incubator at 100 rpm for 6 days at 30°C. Following the termination of fermentation, the culture broth was filtered through Whatman No.1 filter paper at 4°C and filtrate recovered. The cell-free filtrate was used to assay for protease activity as described earlier.

Effect of concentration of carbon source

The effect of concentration (0.1 to 5% w/v) of the best carbon source (glucose) on protease production was evaluated. Two agar plugs (1.7 cm in diameter) of profuse growth of the *Aspergillus* culture cultivated on PDA for 96 h at room temperature were inoculated into 250 ml Erlenmeyer flasks each containing 100 ml sterile basal fermentation medium with varying concentration (0.5 to 6%, w/v) of glucose. Fermentation was carried out in an orbital shaking incubator at 100 rpm for 6 days at 30°C. After the fermentation process, the culture broth was filtered through Whatman No.1 filter paper at 4°C and filtrate recovered. The cell-free filtrate was used to assay for protease activity as described earlier.

Effect of concentration of peptone

The supplementation of the best nitrogen source (soybean meal) with different concentrations (0.1 to 5% w/v) of peptone was equally carried out to determine the best concentration of peptone to be employed for optimal production of the protease. Agar plugs (two agar plugs of 1.7 cm in diameter) of profuse growth of the *Aspergillus* culture cultivated on PDA for 96 h at room temperature were inoculated into 250 ml Erlenmeyer flasks each containing 100 ml sterile basal fermentation medium with varying concentration (0.1 to5%, w/v) of peptone. Fermentation was carried out in an orbital shaking incubator at 100 rpm for 6 days at 30°C. At the end of fermentation, the culture broth was filtered through Whatman No.1 filter paper at 4°C and filtrate recovered. The cell-free filtrate was used to assay for protease activity as described earlier.

Effect of divalent metal ions

The effect of different metal ions on protease production was determined by the addition of the corresponding ion at a final concentration of 0.1% to the production medium. The following salts $FeSO_4.7H_2O$, $MgSO_4$, $ZnSO_4$, $CaCl_2.2H_2O$, $NiSO_4.7H_2O$, $MnSO_4.7H_2O$, $BaCl_2.2H_2O$ and $CoSO_4.7H_2O$ were individually substituted for $MgSO_4.7H_2O$ employed in the basal medium [Medium A] and separately evaluated at concentration of 0.1%. Two agar plugs (1.7 cm in diameter) of profuse growth of the *Aspergillus* culture cultivated on PDA for 96 h at room temperature were inoculated into 250 ml Erlenmeyer flasks each containing 100 ml sterile basal fermentation medium. Fermentation was carried out in an orbital-shaking incubator at 100 rpm for 6 days at 30°C. Following the termination of fermentation, the culture broth was filtered through Whatman No.1 filter paper at 4°C and filtrate recovered. The cell-free filtrate was used to assay for protease activity as described earlier.

Effect of concentration of divalent metal ions

The effect of concentration (0.01 to 0.1% w/v) of the best metal ion ($FeSO_4.7H_2O$) was studied. Two agar plugs (1.7 cm in diameter) of profuse growth of the *Aspergillus* culture cultivated on PDA for 96 h at room temperature were inoculated into 250 ml Erlenmeyer flasks each containing 100 ml sterile basal fermentation medium with varying concentration (0.5 to 6% w/v) of $FeSO_4.7H_2O$. Fermentation was carried out in an orbital-shaking incubator at 100 rpm for 6 days at 30°C. After the fermentation process, the culture broth was filtered through Whatman No.1 filter paper at 4°C and filtrate recovered. The cell-free filtrate was used to assay for protease activity as described earlier.

Effect of initial pH of the medium

The influence of different initial pH values (pH 4.0 to 9.0) of fermentation media on protease production was evaluated at varying pH values. Buffers used are citrate phosphate (pH 4 to 7) and Tris-HCl buffer (pH 8 to 9). The pH values of the fermentation medium were adjusted with digital pH meter (Consort P107) before sterilization by adding 1 M NaOH or 1 M HCl to the required pH.

Effect of surfactants on the production of protease

The influence of 0.1% (v/v) concentration of the following sorbitan derivatives: Tween 40, Tween 65, Tween 80, sodium deoxycholate, cetrimide and Triton X100 on the production of protease was evaluated. The surfactants were separately incorporated into the fermentation medium. Fermentation was carried out in an orbital shaking incubator at 100 rpm for 6 days at 30°C. Following the termination of fermentation, the culture broth was filtered through Whatman No.1 filter paper at 4°C and filtrate recovered. The cell-free filtrate was used to assay for protease activity as described earlier.

Time course of protease production

The time course of protease production was studied by growing *A. carbonarius* in a fermentation medium deduced from the aforementioned nutritional studies, hereafter called medium N. The fermentation medium consisted of 5% soybean meal, 0.1%

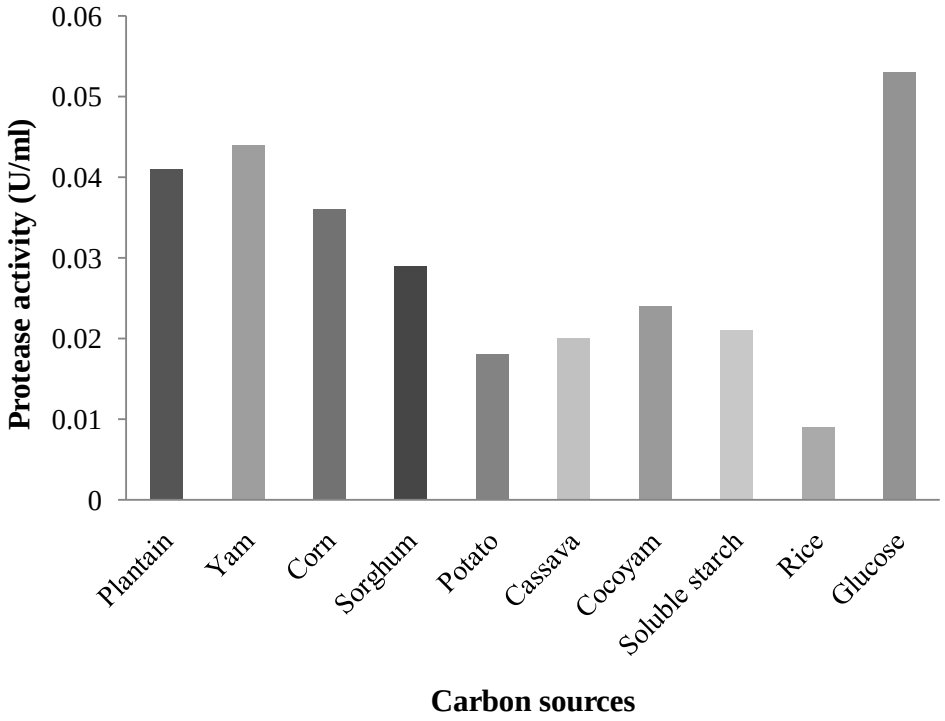

Figure 1. Effect of various carbon sources on protease production by *Aspergillus carbonarius* (Medium A was used for this experiment).

peptone, 5% glucose, 0.04% $FeSO_4.7H_2O$, 0.1% NaCl, 0.1% (v/v) Tween 80 and pH 6.0 in de-ionized water (Medium B). The medium also contained 0.05 mg/ml of chloramphenicol. The medium was autoclaved at 121°C for 20 min at 15 psi. Erlenmeyer flasks (250 ml) each containing 50 ml of fermentation medium N were inoculated with two agar plugs (1.7 cm in diameter) of profuse growth of 96 h old culture of *A. carbonarius*. Fermentation was carried out in an orbital-shaking incubator at 100 rpm for 14 days at 30°C. Following the termination of the fermentation at 24 h intervals, the culture broth was filtered through filter paper (Whatman No.1) and the protease activity of the filtrate, starch disappearance, protein content, biomass (gram dry weight) and pH measured for 336 h (14 days).

Protein concentration estimation

Total protein concentrations were estimated by the Bradford (1976) method using bovine serum albumin (BSA) as standard.

Glucose concentration

Glucose disappearance in the medium was estimated according to DNS methods (Miller, 1959).

Determination of mycelial biomass

The biomass concentration was determined by dry-weight measurement. Culture broth samples (100 ml) were filtered through Whatman No.1 filter paper, washed with distilled water and dried at 80°C for 48 h within which constant weights were obtained.

Statistical analysis

The statistical method used in this study to evaluate all parameters considered was two-way analysis of variance (Cohen, 1985), and student t-test. The least significant difference test was used to identify means that differed significantly. All data, unless otherwise indicated, are results of duplicate experiments.

RESULTS

A clear and distinct zone of protease hydrolysis was observed in the milk agar medium inoculated with *A. carbonarius* (Bainier) IMI 366159 after 96 h of incubation at 30°C and subsequent flooding of the plates with 10% tannic acid. This observation confirms our earlier assumption that this fungus could possess proteolytic activity. Hence, *A. carbonarius* possesses the ability to elaborate protease which hydrolyses casein and other proteinacious substrates. This ability spans through the first 24 h to the 14th day of fermentation.

Effect of carbon sources

Protease productivity of *A. carbonarius* was compared among media containing different types of carbohydrates. Carbon sources greatly influence protease production. The result in Figure 1 shows that the fungus exhibited significantly ($p < 0.001$) higher productivity of protease in

Table 1. Effect of concentration of glucose as carbon source.

Concentration (%)	Protease activity (U/ml)
0.1	0.044
0.5	0.045
1.0	0.047
1.5	0.048
2.0	0.048
2.5	0.038
3.0	0.036
3.5	0.024
4.0	0.026
4.5	0.030
5.0	0.065
5.5	0.063
6.0	0.058

the liquid medium containing glucose compared with other carbon sources. This was closely followed by yam starch. Our result also shows that protease production by this organism has a direct relationship with its growth on different carbon sources evaluated in this study.

The effect of different concentrations of glucose which was the best carbon source was compared as depicted in Table 1. The concentration of 5% glucose significantly ($p < 0.001$) gave the highest yield of protease. It was equally observed that initially the production of protease increased with increase in the concentration of glucose but declined between 2.5 to 4.5% with sharp increase at 5% glucose concentration before declining beyond this concentration. Therefore, concentration of 5% glucose was chosen for the optimum concentration for the protease production by *A. carbonarius*.

Effect of nitrogen sources

Protease productivity of the organism was compared among media containing various types of organic and inorganic nitrogen sources. Figure 2 shows the results of the influence of various nitrogen sources on protease production. The result shows that the fungus exhibited significantly ($p < 0.001$) much higher protease production in the presence of soybean compared with other nitrogen sources.

Our result revealed that the fungus grows better and secretes higher protease when grown on organic nitrogen than inorganic nitrogen sources. The effect of incurporation of 1.5% peptone into the medium containing different nitrogen sources was also compared. The addition of peptone to the medium significantly ($p < 0.001$) enhanced production of protease as shown in Figure 3. The result indicated that soybean incorporated with 1.5% peptone had significantly ($p < 0.001$) higher protease yield than other combinations compared, whereas casein had significantly ($p < 0.001$) the lowest utilization by the fungus on the production of protease. Soybean meal was therefore, chosen as the best nitrogen source for the production of copious amount of protease by the raw starch producing *A. carbonarius*.

Table 2 shows the influence of concentration of soybean meal on protease production. The result shows that the medium containing 5% (w/v) soybean meal was most useful for protease production by the fungus. It is equally evident from the table that high concentration of soybean meal seems to favour protease production than when low concentration of soybean meal is used. Analysis of variance data indicate that the concentration of soybean meal elicited very significant effect at $p < 0.001$ on protease production by *A. carbonarius*.

The result of the effect of varying the concentrations of peptone at 5% soybean meal is depicted in Table 3. The result reveals that highest protease activity was observed at concentration of 5% peptone, followed closely by 0.1% peptone. But since production cost of enzyme highly depends on the cost of medium of production, 0.1% peptone is more economical and cost effective, and therefore chosen for maximum production of protease by *A. carbonarius*. The result of the analysis of variance reveals that concentration of protease is not significant at $p < 0.05$.

Effect of initial pH of the medium

One of the most important environmental factors affecting the growth of fungi is the pH. In this study, 1 M NaOH and 1 M HCl were used to adjust the initial pH of the medium in order to investigate the influence of pH on protease producing ability of the fungus. Figure 4 shows that initial pH 6.0 was the best pH for the production of protease by *A. carbonarius* although protease activity was present at all pHs evaluated in this study. The initial pH of the medium significantly ($p < 0.001$) influenced the production of protease by the organism as confirmed by

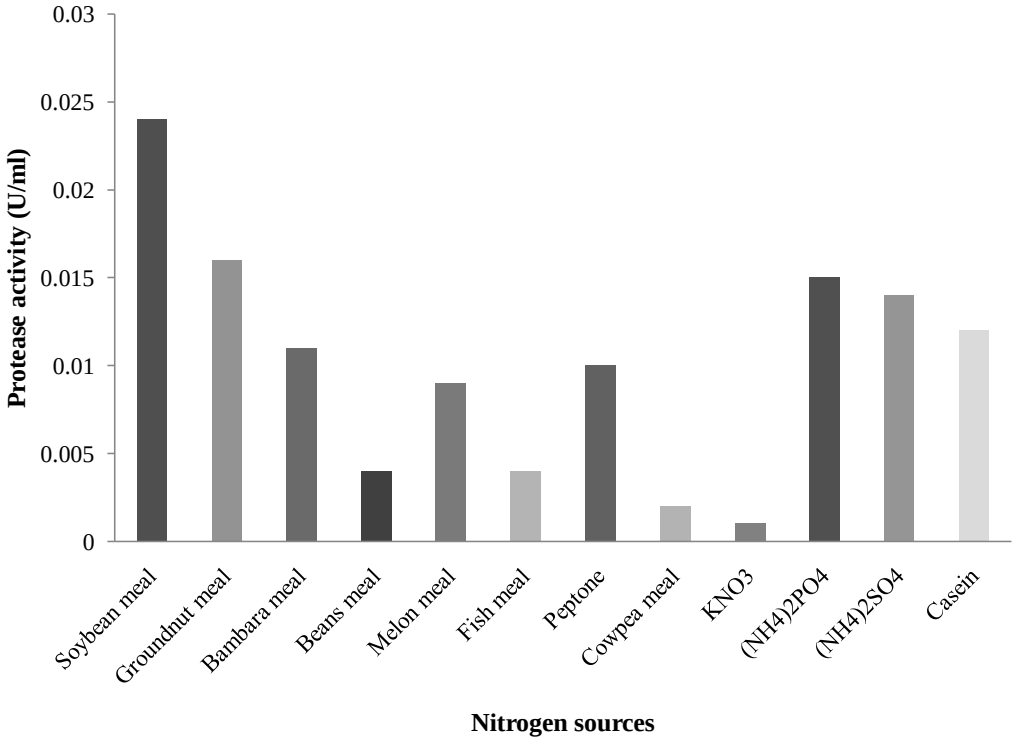

Figure 2. Effect of various nitrogen sources on protease production by *A. carbonarius* (Medium A was used for this experiment).

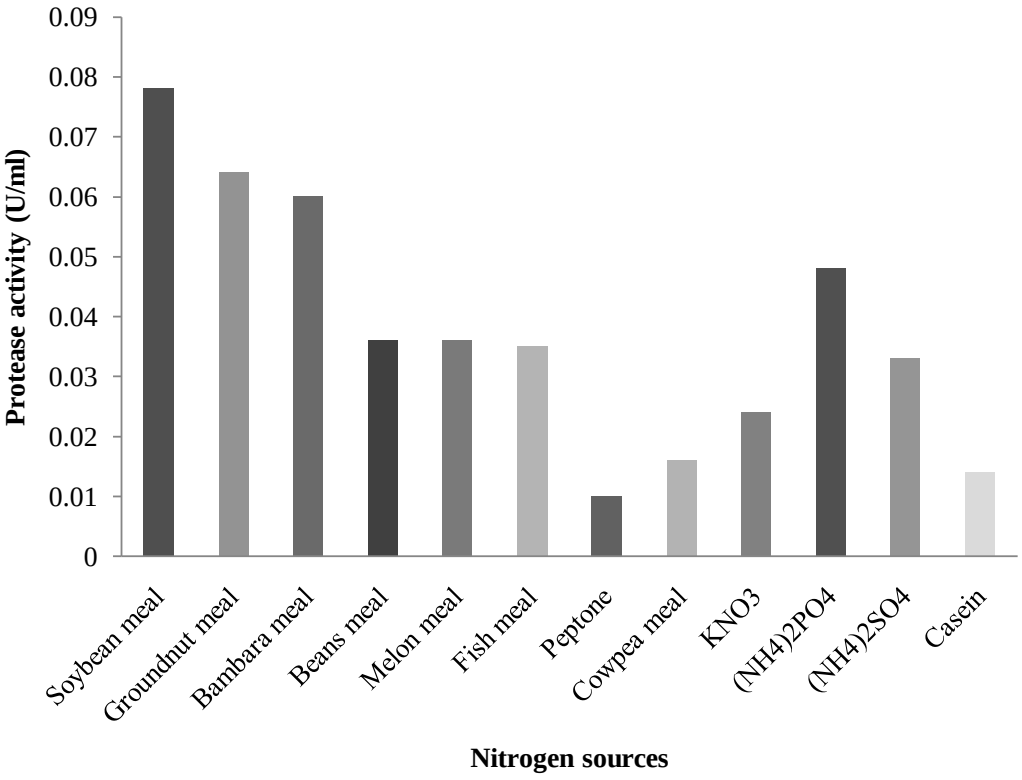

Figure 3. Effect of nitrogen sources incorporated with 1.5% peptone on protease production by *A. carbonarius* (Medium A was used for this experiment).

Table 2. Effect of concentration of soybean meal as nitrogen source on protease production.

Concentration (%)	Protease activity (U/ml)
0.5	0.017
1.0	0.021
1.5	0.020
2.0	0.019
2.5	0.024
3.0	0.029
3.5	0.029
4.0	0.035
4.5	0.049
5.0	0.064
5.5	0.062
6.0	0.056

Table 3. Effect of peptone concentration with 5% soybean on protease production.

Concentration (%)	Protease activity (U/ml)
0.1	0.05
0.5	0.040
1.0	0.029
1.5	0.036
2.0	0.037
2.5	0.027
3.0	0.036
3.5	0.042
4.0	0.044
4.5	0.049
5.0	0.054

the result of analysis of variance.

Effect of divalent metal ions

The results of the effects of metal ions on the production of protease by *A. carbonarius* are shown in Figure 5. The metal ions significantly ($P < 0.001$) influenced protease production by *A. carbonarius*. The result indicates that the highest protease secretion was obtained in medium containing $FeSO_4.7H_2O$ and followed by $CoSO_4.7H_2O$ whereas $MgSO_4.7H_2O$ showed the least effect on the elaboration of protease by the fungus.

Furthermore, Table 4 shows the result of the influence of varying concentration of $FeSO_4.7H_2O$ on production of protease by *A. carbonarius* in submerged fermentation. As illustrated in Table 4, protease production varies with concentration of $FeSO_4.7H_2O$ in the medium. Data presented indicate that protease production was highest when 0.04% of $FeSO_4.7H_2O$ was used in the medium. Analysis of variance data confirm that the concentration of $FeSO_4.7H_2O$ significantly ($p < 0.001$) influenced protease production by *A. carbonarius.*

Effect of surfactants on production of protease

Generally, surfactants play a major role in the secretion of enzymes in fungi. The result of the effect of 0.1% (w/v, v/v) of various surfactants on production of protease by *A. carbonarius* is shown in Figure 6. It was observed that medium containing Tween 80 had the highest positive influence on production of protease compared to other surfactants evaluated whereas sodium desoxycholate medium elicited lowest amount of protease production by the fungus. The presence of surfactant (Tween 80) in the medium significantly ($p < 0.001$) influenced protease production.

Time course of protease production by *A. carbonarius*

The result of the profiles of the mycelial growth, protease

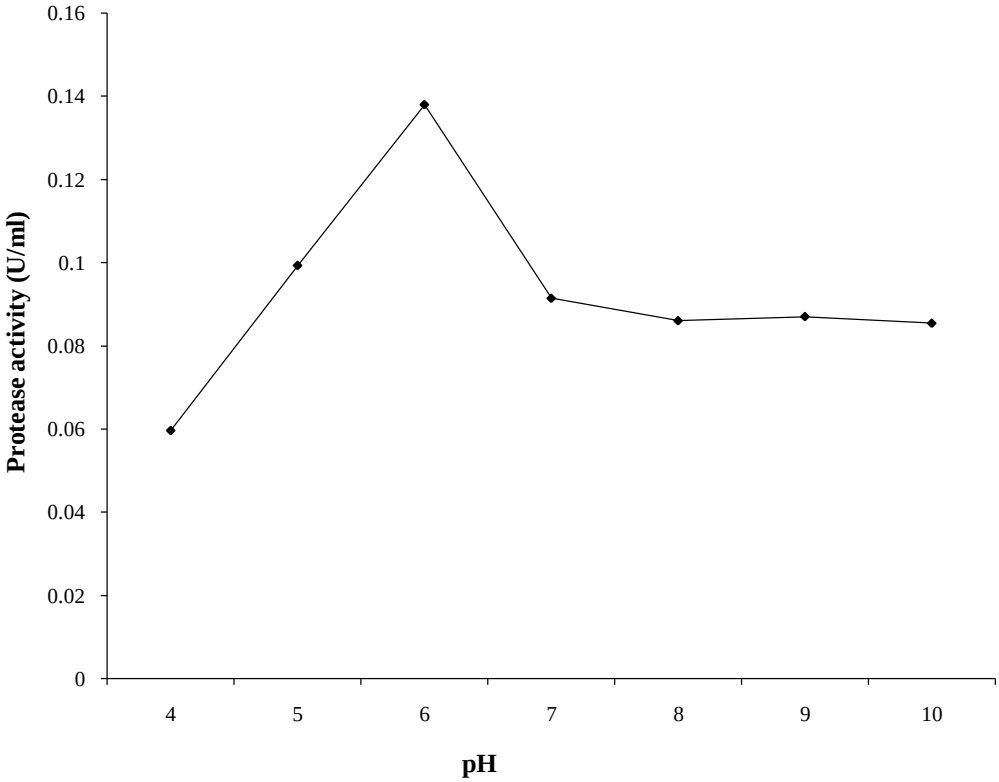

Figure 4. Effect of initial pH on protease production by *A. carbonarius* in submerged culture (Medium A was used for this experiment).

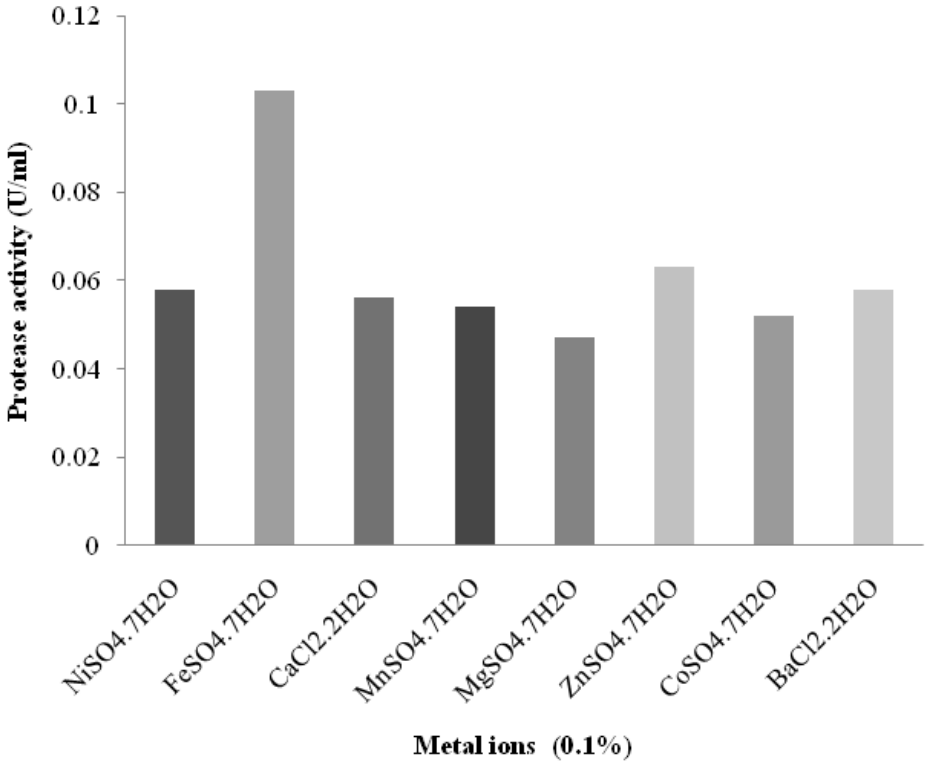

Figure 5. Effect of metal ions on protease production by *A. carbonarius* (Medium A was used for this experiment).

Table 4. Effect of concentration of $FeSO_4.7H_2O$ on the production of protease.

Concentration (%)	Protease activity (U/ml)
0.01	0.080
0.02	0.082
0.03	0.096
0.04	0.110
0.05	0.094
0.06	0.079
0.07	0.074
0.08	0.046
0.09	0.053
0.10	0.074

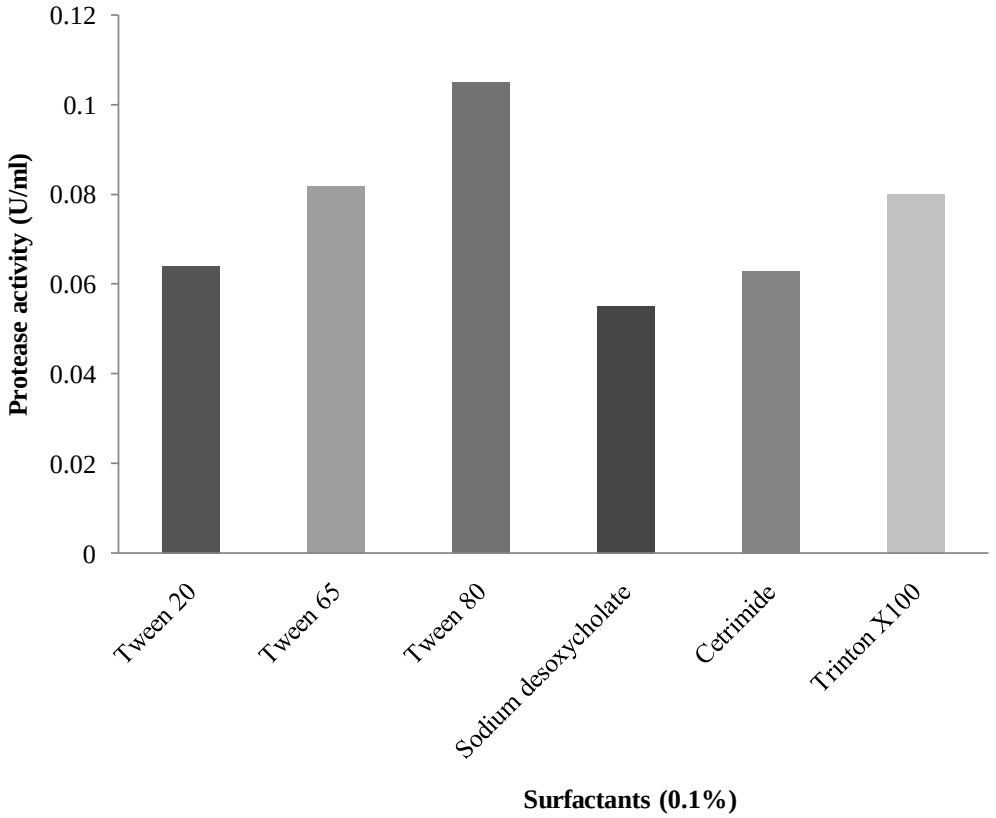

Figure 6. Effect of surfactants on the protease production by *A. carbonarius* (Medium A was used for this experiment).

activity, glucose concentration and protein content under optimal condition is depicted in Figure 7. The optimal medium for the production of protease by *A. carbonarius* was: 5% soybean meal, 0.1% peptone, 5% glucose, 0.04% $FeSO_4.7H_2O$, 0.1% NaCl, 0.1% (v/v) Tween 80 and pH 6.0. Maximum protease activity was obtained on the 9th day of cultivation with shaking at 100 rpm in a 250 ml cotton-plugged Erlenmeyer flask containing 50 ml of the medium at 30°C. As illustrated in Figure 7 there was a fall in pH from initial pH 6.0 of the fermentation medium to pH 4.0 within 24 h of fermentation process and then remained fairly stable within this range throughout the period of cultivation.

The result also shows that the level of glucose in the fermentation medium for the production of protease by *A. carbonarius* decreased sharply within 72 h. Glucose consumption by the fungus increased quite slowly from day 3 to day 14 of the fermentation period. Data presented in Figure 7, indicate that the biomass increased from day 1 to day 6 beyond which there was a

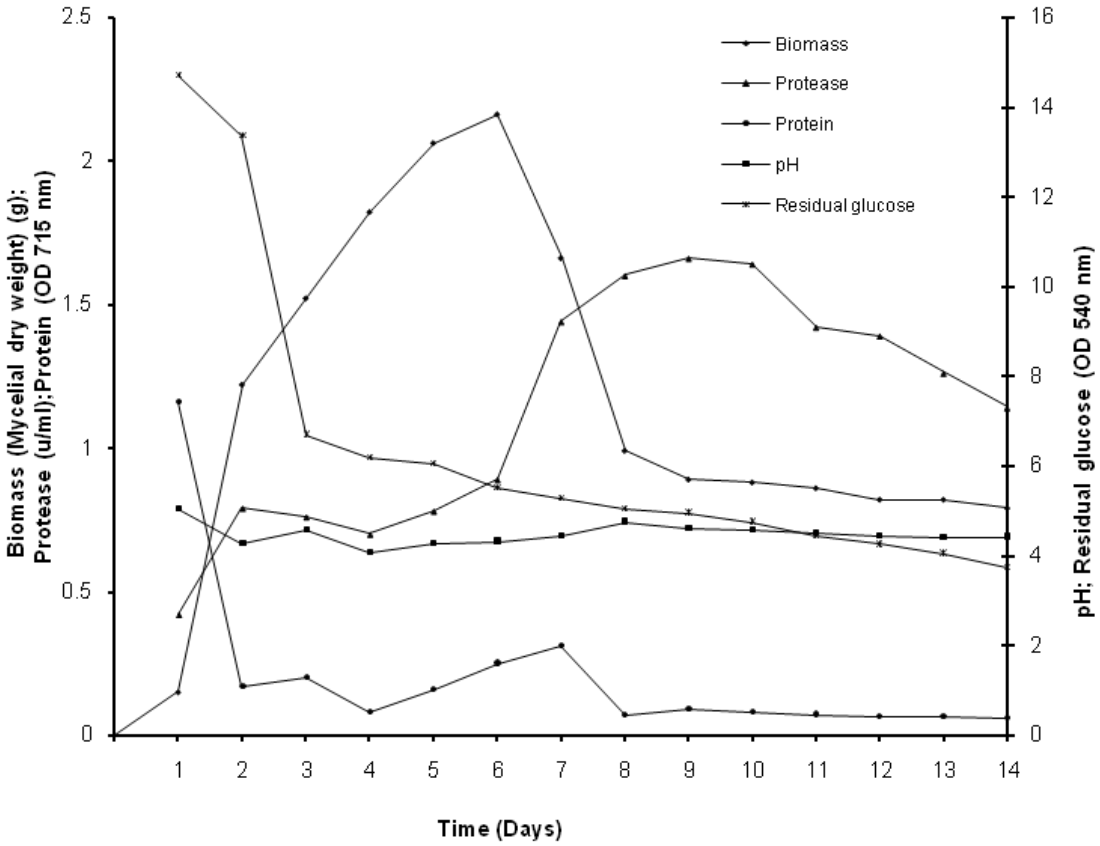

Figure 7. Time course of protease production by *A. carbonarius* in submerged culture (Medium B was used for this experiment).

sharp decrease in cell growth. Protease activity increased through the end of the culture with the growth of the fungus reaching the highest activity on the 9th day of cultivation. It is observed from the result that a rapid increase in protease activity began at the end of the exponential growth phase. Thereafter protease activity decreased gradually with cell autolysis. Our result shows that there is direct relationship between time of growth and extracellular protease production.

DISCUSSION

A. carbonarius grew and produced significantly ($p < 0.001$) appreciable level of protease using glucose and soybean meal in combination with 0.1% peptone as carbon and nitrogen sources, respectively. Growth and protease production by this fungus were significantly ($p < 0.001$) influenced by the type and concentration of the carbon and nitrogen sources used in the medium (Mamo and Gessesse, 1999). Presently, about 60% of total industrial enzyme market is accounted for by proteases extensively used for leather, pharmaceutical, detergent, tanning, and food applications (Gessesse et al., 2003). Up to 30 to 40% of the production cost of industrial enzymes is accounted for by the growth substrates (Hinman, 1994; Kalisz, 1988). Subsequently, the use of cheap and readily available substrates could lead to drastic reduction in the production cost of proteases (Gessesse et al., 2003). Data presented in this study show that the absence of glucose results in a dramatic decrease in enzyme production. There are reports that the presence of glucose in a culture medium suppressed protease production (Sen and Satyanarayana, 1993). This is contrary to our observation as the presence of glucose in the production medium elicited the highest protease activity among other carbon sources evaluated and is consistent with other observations (Dahot, 1993; Gajju and Bhalla, 1996; Adinarayana and Ellaiah, 2002; Andrade et al., 2002; Papagianni and Moo-Young, 2002). Our result is in agreement with Srinubabu et al. (2007a) and Srinubabu et al. (2007b) who reported glucose as the best carbon source for the production of protease by *Aspergillus oryzae*.

The stimulatory effect of glucose on protease production at and beyond 5% (w/v) glucose concentration indicates that protease production by *A. carbonarius* may not be subject to control by catabolite repression. This

observation contradicts the glucose mediated repression of protease production advanced by many authors (Sen and Satyanarayana, 1993; Miller, 1980; Ferrero et al., 1996). The type of nitrogen sources used for cultivation of the *A. carbonarius* also affected protease production. Although it has been reported that the production of proteases in complex growth media usually promotes exuberant growth and high protease yield (Joo et al., 2002), their expensive cost makes them unsuitable for a large-scale production. The use of cost-effective media for enzyme production is especially relevant as 30 to 40% of the production cost of industrial enzymes is dependent on the cost of the growth media (Joo et al., 2003). In this study, among the various organic and inorganic nitrogen sources, the maximum protease activity was obtained when soybean meal was used as sole nitrogen source. Soybean meal is a very common and inexpensive source of nitrogen (Margesin et al., 2005) and this will go a long way in minimizing the cost of production of protease from this organism. Soybean meal is inexpensive and readily available because it is largely produced as a by-product during oil extraction (Joo et al., 2002). The finding is consistent with the reports of other on the significant enhancement of protease production from *Pedobacter cryoconitis*, *Bacillus clausii* and *Bacillus horikoshii*, respectively with soybean meal (Wang and Shih, 1999; Joo et al., 2002; Margesin et al., 2005). Srinubabu et al. (2007a) reported that soya bean meal significantly induced the production of protease in *Aspergillus oryzae*. The supplementation of soybean meal medium with peptone stimulated higher protease production. It was observed that protease elaboration by this fungus increased with increase in the concentration of soybean meal and peptone medium. But for economic reasons, 0.1% peptone which equally elicited high protease production was chosen to minimize production cost of the enzyme.

Divalent metal ions are required in the fermentation medium for optimum production of proteases. Cells may require divalent ions for the production (Damare et al., 2006) or activity (Beynon and Bond, 1989) of proteases. The requirements for specific divalent metal ions however, depend on the source of enzyme. Our data show that iron sulphate ($FeSO_4.7H_2O$) favoured highest production of the protease compared to other ones evaluated. The least protease production was observed in medium containing magnesium sulphate ($MgSO_4.7H_2O$). Addition of metal ions to growth media has been shown to increase protease activity and is also a requirement for production and stabilization of some proteases (Olson and Ohman, 1992; Rao et al., 1998; Damare et al., 2006). Presence of surfactants in production medium exerts great influence in the secretion of enzymes in fungi (Rao et al., 1998). Surfactants alter cell permeability of microorganisms which lead to increased protein secretion or surface effects on cell-bound enzymes. The addition of Tween 80 increased the protease production by several folds. The result of this study therefore, revealed that Tween 80 at a concentration of 0.1% was the best surfactant for optimum production of the protease. This is consistent with the observation that there was an elevation of protease production by *Aspergillus ustus* on supplementation with Tween 80 to the fermentation medium (Kohlman et al., 1991). The least protease production was observed in medium containing 0.1% sodium deoxycholate. Protease production by microbial strains depends on the extracellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production (Ellaiah et al., 2002; Kumar et al., 2008). Protease production by this fungus was influenced by the initial pH of the medium. The best protease production was observed when the initial pH was 6.0. The result of this study is consistent with the observation of Vishwantha and Appu (2010) who reported pH 5.0 as the best initial pH for the production of protease from *Aspergillus oryzae* MTCC 5341.

The time course for biomass, glucose consumption, pH, extracellular protease secretion by *A. carbonarius* cultivated in shake flasks are shown in Figure 7. The extracellular protease secretion commenced after 24 h of culture. Data presented in Figure 7 revealed that protease was produced throughout the 14 day fermentation period. Extracellular protease production commenced after 24 h of inoculation of the fungus and increased up to the ninth day after which it decreased. However, maximum protease production occurred at day 9 of the fermentation. It has been reported that *A. ustus* (NIOCC #20) maximum protease production occurred on the 7th day of cultivation (Kohlman et al., 1991). The study equally revealed that the organism effectively utilized the protein constituents in the fermentation medium within 24 h of cultivation. Thereafter, it commenced secretion of its own protein and had maximum total protein production at day 7 after which it declined while the maximum protease occurred on the 9th day for cultivation. Protease production increased when the fungus had optimum secretion of its total protein.

The result revealed that the intracellular protease was secreted mainly after the cell growth approached stationary phase. This pattern is indicative of a secondary metabolite. Our result revealed that there is a direct relationship between time of growth and extracellular protease secretion. Protease produced in the medium in early growth phase may decrease in the later phase of growth probably due to the degradation of neutral protease by alkaline protease during later phase of growth (Dahot and Memon, 1989). This may lead to protease formed at different growth phases to differ in their chemical nature. In this study, we have established the optimum condition for production of protease by *A. carbonarius,* although there may not have been any established defined medium for the optimum production

of protease from various microbial sources. Each organism seems to possess its own peculiar condition for optimum protease production. Different carbon and nitrogen sources elicited varying effects on protease. As depicted in Figure 1 glucose induced the highest protease activity followed by yam starch while the lowest protease production was observed in cultures with raw rice starch as sole carbon source.

Conclusion

The optimization of medium composition is done to maintain a balance between the diverse medium constituents in order to tame the amount of unutilized components at the end of fermentation. The optimal fermentation medium for the production of protease by *A. carbonarius* (Bainier) IMI 366159 was 5% of soybean meal, 0.1% peptone, 5% glucose, 0.04% $FeSO_4.7H_2O$, 0.1% NaCl, 0.1% (v/v) Tween 80 and pH 6.0.The results presented in this work therefore, suggests the possibility of secretion of protease by *A. carbonarius* using cheaper and locally available substrates as carbon and nitrogen sources and its subsequent application in industries.

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